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Purification and partial characterization of a cholinergic neuronal differentiation factor

(phenotypic choice/sympathetic neuron/development/cardiac cell)

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ABSTRACT **The choice of transmitter made by postmitotic rat sympathetic neurons in cell culture can be controlled by the environment in which they develop. One of the differentiation signals is a protein secreted by heart cells that can induce previously noradrenergic neurons to synthesize acetylcholine and form cholinergic synapses. This change in phenotype occurs without alteration in neuronal survival or growth. The differentiation factor has now been purified at least 100,000-fold, and it is homogeneous by several criteria. (*i*) The cholinergic activity comigrates with a single ^{125}I-labeled protein band of 45 kDa in one-dimensional NaDodSO$_4$/PAGE. (*ii*) The biological activity comigrates precisely with a series of five ^{125}I-labeled protein spots of 45 kDa in two-dimensional gel electrophoresis. (*iii*) Treatment of the 45-kDa band with endo-β-*N*-acetylglucosaminidase F reduces the apparent molecular size of both the labeled protein and the biological activity to a band of 22 kDa. The data suggest that the differentiation factor is a slightly basic glycoprotein with at least six glycosylation sites.**

Differentiation of appropriate phenotypes in the developing vertebrate nervous system involves a high degree of cellular interaction. Perhaps the most detailed information concerning environmental influences on differentiation involves the choice of neurotransmitter and synaptic phenotype made by neural crest derivatives (1–4). In these studies, the decision under investigation is not whether to differentiate or not, but rather which of many possible differentiation paths to take. Single postmitotic rat sympathetic neurons, for instance, can be made to form noradrenergic, cholinergic, or dual-function synapses with cardiac myocytes in microcultures (5–7). Depending on the developmental cues present *in vitro*, these neurons also have the capacity to produce several neuropeptides (8) and purines (9, 10), and they can be induced to form electrical synapses (11–13). This plasticity in choice of transmitter is not simply a reflection of the developmental potential these neurons possess under artificial conditions, because at least some of these phenotypic transitions are actually expressed during normal embryogenesis. That is, the subpopulation of sympathetic neurons that is cholinergic and innervates the sweat glands in the rat passes through a noradrenergic stage before becoming cholinergic (14, 15). Substitution of one transmitter or neuropeptide phenotype for another during normal development may also occur in mammalian enteric, sensory, and parasympathetic ganglia (16–19); in retina (20); and in identified snail neurons (21).

The culture paradigm offers an opportunity for identifying and characterizing some of the environmental signals that can influence these differentiation decisions. In the case of the sympathoadrenal lineage, a number of such cues have been identified, including nerve growth factor (22–24), glucocorticoids (22–27), epidermal growth factor (26), insulin (12, 13), electrical activity and Ca^{2+} influx (28, 29), rat serum (30), and nonneuronal cells and their conditioned medium (CM) (31–34). Such signals have been termed "instructive," in order to distinguish them from growth factors that have been termed "permissive" (2). Some developmental cues can act in either way, however, depending on the circumstances. For instance, nerve growth factor is permissive for the development of both noradrenergic and cholinergic sympathetic neurons (35), but it can also act instructively by converting chromaffin cells into neurons (22, 23). Similarly, glucocorticoids and insulin are necessary for the survival of certain cells in the sympathoadrenal lineage, but these hormones can also influence the choice of phenotype as well (12, 13, 22–26).

There have been many reports of macromolecules influencing neuronal development, but in most cases it has been difficult to assess the instructive vs. permissive effects of these agents on their target neurons. One clear case of a selective differentiation effect is the release of a factor by certain nonneuronal cells that can convert noradrenergic sympathetic neurons to the cholinergic phenotype without altering survival or growth (31, 32, 34, 36). The same, or a very similar, factor induces cholinergic properties in sensory and spinal cord neurons (37, 38) and, possibly, in parasympathetic neurons as well (39). Direct comparison of these factors depends on purifying and characterizing them. Studies on their roles in normal development *in vivo* also require purification and the generation of appropriate immunological and molecular probes. Purification of differentiation factors in CM has proven difficult because of the small amounts of material available and the long assay time required (2–3 weeks). Progress has been made with the cholinergic factor that acts on sympathetic neurons, however. Initial characterization of this molecule, obtained from serum-containing CM, revealed that the activity resides in a Pronase-sensitive molecule of 45 kDa, as determined by Sephadex chromatography (40). Manipulation of the hormonal composition of the medium enabled me to obtain an active cholinergic preparation of serum-free CM from neonatal rat heart cells (26). By using this starting material, an apparently homogeneous glycoprotein has now been obtained that retains the ability to both induce cholinergic properties and reduce adrenergic properties in cultures of dissociated sympathetic neurons from the neonatal rat superior cervical ganglion. This report presents the purification protocol and describes some of the properties of this protein. Preliminary accounts of this work have appeared previously (41–43).

MATERIALS AND METHODS

[*methyl*-^{3}H]Choline chloride and L-[2,6-^{3}H]tyrosine were obtained from New England Nuclear or from Amersham. *N*-succinimidyl 3-(4-hydroxy, 5-[^{125}I]iodophenyl)propionate

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Abbreviations: AcCho, acetylcholine; CA, catecholamine.

(^{125}I-Bolton–Hunter reagent) was obtained from Amersham. Endo-β-*N*-acetylglucosaminidase F (endoglycosidase F) was obtained from New England Nuclear. 1,3,4,6-Tetrachloro-3α-6α-diphenylglycouril (Iodo-Gen) was from Pierce.

Neuron Cultures. Neuron cultures were prepared as described by Hawrot and Patterson (44). The fractions to be tested for biological activity were kept frozen in sterile aliquots after dialysis against phosphate-buffered saline. The fractions were mixed with fresh growth medium (44) and given to the cultures every other day. Neurotransmitter production was assayed on day 16–19.

Purification of the Cholinergic Factor. Serum-free hormone-supplemented CM from primary cultures of newborn rat heart cells was obtained as described (26). The first purification step was precipitation with ammonium sulfate as described by Weber (40). All the activity was recovered in the 60–100% precipitate. DEAE and CM-cellulose and Sephadex column chromatography were performed as described by Weber (40) with minor modifications. All the activity ran through DEAE-cellulose columns equilibrated with 5 mM sodium phosphate, pH 7.0/0.2 mM Na_2EGTA (DEAE-cellulose fraction). The DEAE-cellulose fraction was applied to a CM-cellulose column and eluted with a gradient of 5–100 mM sodium phosphate, pH 7.0/0.2 mM Na_2EGTA/0.01% polyethylene glycol 6000 (PEG). PEG was included in all buffers after this stage of purification to prevent loss of the activity due to nonspecific adsorption. More than 80% of the activity was recovered as a single peak at 15–40 mM salt concentration (CM-cellulose fraction). The behavior on these columns indicates that the cholinergic factor is a slightly basic molecule. The CM-cellulose fraction was loaded on a Sephadex G-100 (Superfine, Pharmacia) column (1.5 × 100 cm) in 5 mM phosphate, pH 7.0/150 mM NaCl/0.2 mM Na_2EGTA/0.01% PEG after concentration on a PM-10 Diaflow membrane (Amicon) and run at a flow rate of ≈3.5 ml/hr. A single activity peak was obtained at an apparent molecular size of ≈45 kDa (Sephadex fraction). The $NaDodSO_4$/PAGE system of Laemmli (45) was used as the final purification step. The separating gel was 10% polyacrylamide made from a stock solution of 30% acrylamide and 0.2% *N,N'*-methylene-bisacrylamide. The *N,N'*-methylene-bisacrylamide concentration was lower than that normally used to improve the elution efficiency. Disulfide reducing agents were not included in the sample buffer except where specified, because they destroy the activity in the presence of $NaDodSO_4$. The molecular size markers used were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (22 kDa), and lysozyme (14.3 kDa). Samples from preparative $NaDodSO_4$/PAGE were tested for biological activity as follows: the gel was sliced into 2- to 3-mm segments and eluted overnight at 4°C in 0.05 M Tris acetate, pH 7.8/0.01% PEG/bovine serum albumin (2 mg/ml). Each eluate was loaded on an AG 1-X2 (Bio-Rad) column equilibrated with 0.05 M Tris acetate, pH 7.8/0.01% PEG to remove $NaDodSO_4$ according to the method of Weber and Kuter (46). The run-through fraction was dialyzed against phosphate-buffered saline, sterilized by filtration, and kept frozen in aliquots until use.

Two-Dimensional Gel Electrophoresis. Nonequilibrium pH gradient electrophoresis for both basic and acidic proteins was performed as described by O'Farrell *et al.* (47) with the following modifications. Samples were concentrated and desalted by centrifugation on centricon filters (Amicon). Urea, Nonidet P-40, and ampholines (LKB) were added to final concentrations of 9.5 M, 2%, and 2%, respectively, and loaded as the first-dimension gel (0.25 × 11 cm). Ampholines (pH 3.5–10) or a mixture containing equal parts of ampholines at pH 6–8 and pH 5–7 were used. Samples were electrophoresed, usually for 4–5 hr at 400–500 V, for a total of 2000–3000 V·hr in the first dimension. The first-dimension cylindrical gel was immediately loaded on the second-dimension slab gel without equilibration to avoid protein loss, as described by O'Farrell (48), and it was run as described above. Standards used were bovine serum albumin (68 kDa; pI, 4.9), ovalbumin (43 kDa; pI, 4.6), myoglobin (17.5 kDa; pI, 7.0), and cytochrome *c* (13.3 kDa; pI, 10.7).

Endoglycosidase F Treatment. Incubations were carried out for 16 hr at 37°C in buffer containing 0.1 M sodium phosphate, pH 6.0/50 mM EDTA/1% Nonidet P-40/0.1% $NaDodSO_4$/1 mM phenylmethylsulfonyl fluoride/0.01% PEG, according to the conditions recommended by New England Nuclear (49). The amount of added endoglycosidase F varied from 0 to 20 units, where 1 unit is the amount of enzyme that deglycosylates 15 μg of RNase B in 1 hr (New England Nuclear). 2-Mercaptoethanol facilitated deglycosylation by endoglycosidase F (49) but did not increase the apparent number of cleavage sites for endoglycosidase F. $NaDodSO_4$ was added to a final concentration of 3% in preparing samples for $NaDodSO_4$/PAGE.

Iodination. Iodination of tyrosine residues was performed by the chloramine-T (50), Iodo-Gen (Pierce), or lactoperoxidase (51) method. Iodination according to the Bolton–Hunter method (52) was performed as follows: 100–200 μg of the Sephadex fraction in 0.1 M borate buffer (pH 8.5) was added to 0.25–0.5 nmol of the dried ^{125}I-Bolton–Hunter reagent and the reaction was allowed to proceed for 15 min on ice. To inactivate unreacted reagent, glycine was added to a final concentration of 0.25 M and the mixture was kept at 4°C until loading on the gel.

Silver Staining. Silver staining of proteins separated by $NaDodSO_4$/PAGE was done according to the methods of Merril *et al.* (53), Irie *et al.* (54), and Oakley *et al.* (55). In these methods, at least 10 ng of ovalbumin was detected as a standard.

Estimation of Protein. The amount of protein was determined by the method of Lowry *et al.* (56), except following $NaDodSO_4$/PAGE. Since the 45-kDa region, where biological activity is located (see below), was not labeled by silver-staining methods, Bolton–Hunter iodination was used to estimate the protein content of this area. The ^{125}I-labeled Sephadex fraction was subjected to 10% $NaDodSO_4$/PAGE after separation from the ^{125}I-Bolton–Hunter reagent by Sephadex G-50, equilibrated with phosphate-buffered saline containing 0.25% gelatin. The percentage of the label in the 45-kDa region was calculated, and the amount of protein was then estimated by multiplying the percentage by the total protein in the ^{125}I-labeled Sephadex fraction applied to the gel. Alternatively, known amounts of ovalbumin were labeled in the same way and analyzed by $NaDodSO_4$/PAGE. The radioactivity in the ovalbumin band of known protein concentration was compared to the radioactivity in the 45-kDa region of the Sephadex fraction. Both methods yielded similar estimates.

RESULTS

Purification of the Factor. The assay for cholinergic activity involves incubating CM fractions with primary cultures of dissociated sympathetic neurons for 2–3 weeks, changing the medium every other day. At the end of this period, the cultures are incubated with [^{3}H]tyrosine and [^{3}H]choline chloride for 4–5 hr, extracted, and the resulting ^{3}H-labeled catecholamine (CA) and acetylcholine (AcCho) were separated by paper electrophoresis and counted. The resulting data are a measure of net synthesis and accumulation of the transmitters from their precursors, and they include the processes of synthesis, storage, and degradation (57). This is a physiologically relevant measure of how the neurons use precursors *in vivo*. Results from such experiments have also

Table 1. Purification of the cholinergic factor

Fraction	Total protein, mg	Total activity, units	Specific activity, units/mg	Recovery, %	Purification, -fold
CM	44,000	40,340	0.9	100	1
$(NH_4)_2SO_4$	43,000	26,120	0.6	65	0.7
DEAE-cellulose	142.0	52,850	370	130	410
CM-cellulose	22.0	32,210	1,460	80*	1,620
Sephadex G-100	6.0	30,000	5,000	74	5,560
$NaDodSO_4$/PAGE	0.07	6,430	91,900	16	102,000

Units of activity are expressed as the transmitter ratio, AcCho/CA (32). The amount of protein was determined as described. Serum-free CM (17.5 liters) was used as the starting material for the results shown here.
*The activity is recovered from the CM-cellulose column as a single broad peak at 15–40 mM salt concentration. However, only some of these fractions were pooled in order to increase the specific activity. Thus, the recovery of 80% is an underestimate.

been shown to correlate very well with the levels of the biosynthetic enzymes for these transmitters (choline acetyltransferase and tyrosine hydroxylase) as determined by activity assays of homogenates and by immunocytochemical staining (32, 58). The data are usually expressed as the increase in the ratio of the two transmitters, AcCho/CA, caused by a particular concentration of CM (-fold induction).

While in this laboratory, Weber (40) developed a number of initial purification steps for the cholinergic factor by using serum-containing CM. After devising a suitable serum-free medium based on that of Bottenstein *et al.* (59), I applied several of these steps in sequence to achieve a 5560-fold increase in specific activity with a high recovery (Table 1). Since the activity is stable to treatment with $NaDodSO_4$ under nonreducing conditions, preparative $NaDodSO_4$/PAGE was added as a final step. As shown in Fig. 1, the activity runs as a single peak of 45 kDa, and this material is purified at least 100,000-fold (Table 1). The estimate of protein values in the most purified fractions is indirect, as mentioned in *Materials and Methods*. The low yield of activity is due to poor recovery of protein during the removal of $NaDodSO_4$ rather than an irreversible denaturation of the protein. The yield of protein in the 45-kDa peak is estimated to be 70 μg, using 17.5 liters of CM as the starting material. The factor is active at concentrations <10 ng/ml, and it displays the ability to both induce AcCho synthesis and reduce CA synthesis.

Protease Sensitivity. Weber (40) found that the activity of the cholinergic factor in serum-containing CM was partially decreased by treatment with a protease from *Streptomyces griseus*, but that the preparation contained large amounts of protease inhibitors, which precluded further analysis. The activity in the Sephadex fraction from serum-free CM is almost completely lost by treatment with trypsin (EC 3.4.21.4; Sigma, type III) [<10% remaining after incubation with trypsin (2.5 μg/ml) at 37°C for 14 hr]. The trypsin was inactivated with turkey egg white trypsin inhibitor after the incubation (40). Thus, the biological activity appears to reside in a protein.

Labeling the Protein. As shown in Fig. 1, silver staining revealed no obvious band corresponding to the activity peak. Iodination of tyrosine residues by the Iodo-Gen, chloramine-T, and lactoperoxidase methods also failed to yield a distinct band at 45 kDa. Iodination of lysine residues by the Bolton–Hunter method, however, did reveal a labeled band at the 45-kDa region (Fig. 2). This protein comigrates with the activity in two further analytical steps.

Two-Dimensional Gel Electrophoresis. Subjecting the ^{125}I-labeled Sephadex fraction to two-dimensional gel electrophoresis (nonequilibrium pH gradient electrophoresis is followed by $NaDodSO_4$/PAGE) reveals five distinct spots in the 45-kDa region (Fig. 3). When the 45-kDa region of the gel is cut into small slices and assayed for both ^{125}I and cholinergic activity, results such as those shown in Fig. 4 are obtained. The yield of biological activity is 60–80% of that obtained in one-dimensional gels. The comigration of the activity and the labeled protein has been seen in all three experiments of this type that have been done. Although each protein spot has detectable activity, the relative intensity of these spots and their relative biological activity vary in different experiments, and the ratio of activity to ^{125}I varies considerably among the spots, with the more acidic species having lower ratios. This variation could be due to different degrees of iodination or to different degrees of inactivation by iodination. Iodination itself, however, is not the cause of the charge differences among the spots, because multiple spots of cholinergic activity are also seen in unlabeled preparations (data not shown). Whatever the reason, the fact that the activity peaks

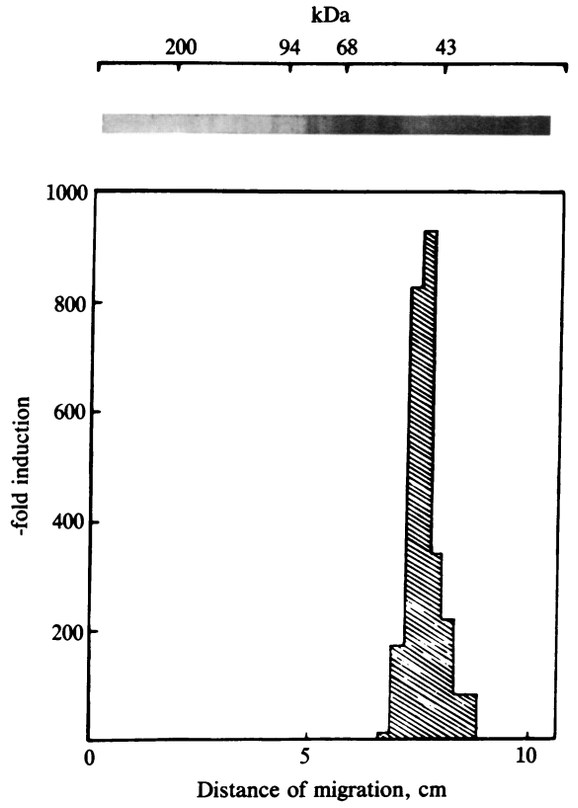

FIG. 1. $NaDodSO_4$/PAGE of the Sephadex fraction. The Sephadex fraction was subjected to $NaDodSO_4$/PAGE and the biological activity eluted from each gel slice was tested. Illustrated on the top part of the figure is a portion of the gel stained with silver by the method of Merril *et al.* (53). There is no clear sharp band at the 45-kDa region corresponding to the activity peak at 45 kDa.

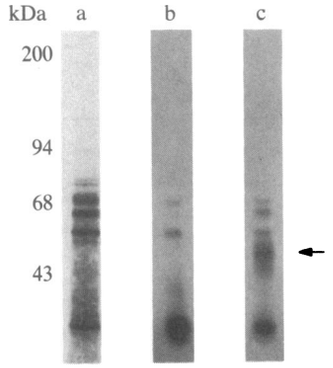

FIG. 2. Labeling of 45-kDa protein. The Sephadex fraction was either stained with silver (lane a), iodinated by the Iodo-Gen method (lane b), or by the Bolton–Hunter method (lane c). The 45-kDa area where activity is located (arrow) is barely stained with silver (visible only in the wet gel). It is not iodinated by the Iodo-Gen method, which labels tyrosine residues, but it is heavily labeled by the Bolton–Hunter iodination method, which primarily labels lysine residues (51).

and the ^{125}I-labeled spots line up precisely strongly suggests that the labeled 45-kDa molecule is the cholinergic factor.

Deglycosylation of the Protein. When the purified 45-kDa band is treated with endoglycosidase F, a series of six new and distinct labeled bands appears, suggesting that there are at least six glycosylation sites on the protein (Fig. 5). Exhaustive deglycosylation converts all of the bands to the species of lowest size, 22 kDa. This presumptive protein core retains just as much biological activity as the 45-kDa band incubated without endoglycosidase F (the increase in the AcCho/CA ratio induced by the 22-kDa form is 1.3 times higher than the increase induced by the 45-kDa form, using equal amounts of ^{125}I-labeled protein in two separate experiments, and cholinergic activity was only found in the 22-kDa band after exhaustive deglycosylation as in Fig. 5, lane e). Thus, deglycosylation causes a shift in apparent molecular size of 23 kDa, and the cholinergic activity again comigrates with the labeled protein.

DISCUSSION

The evidence that the ^{125}I-labeled 45-kDa protein is the cholinergic factor is its comigration with the cholinergic activity after one- and two-dimensional gel electrophoresis and after a large shift in apparent molecular size caused by treatment with the enzyme endoglycosidase F. Final proof of homogeneity requires N-terminal sequence analysis, and our preliminary findings indicate that both the 45-kDa and 22-kDa (deglycosylated by hydrogen fluoride) bands have a single and identical N-terminal amino acid sequence (unpublished data).

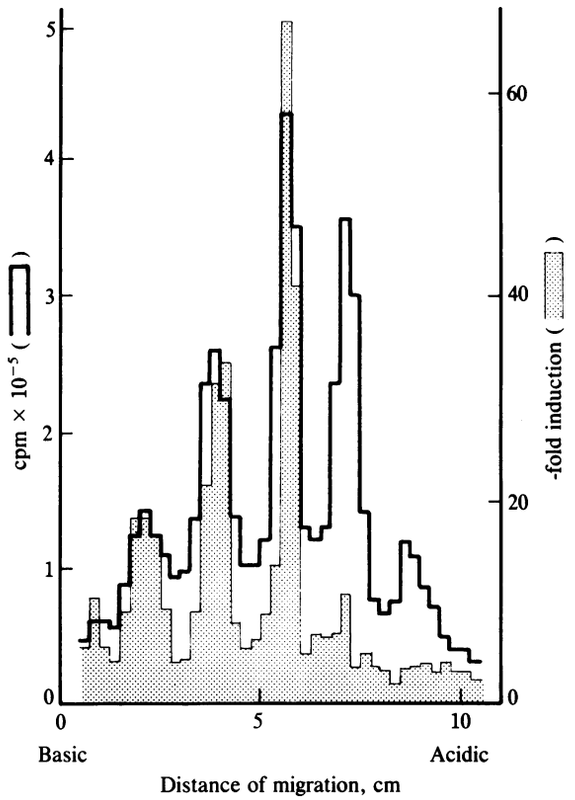

FIG. 4. Cholinergic activity in two-dimensional gel electrophoresis. The 45-kDa region of the two-dimensional gel similar to that shown in Fig. 3 (except that the pH gradient was from 5.7 to 7.5) was cut in 2.5-mm slices and the eluted fraction from each gel slice was counted for ^{125}I radioactivity and tested for biological activity. Each of the activity peaks and the ^{125}I spots line up precisely, indicating that the labeled 45-kDa molecule is the cholinergic factor.

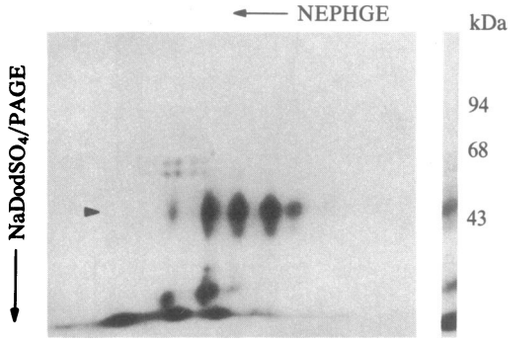

FIG. 3. Two-dimensional gel analysis of the ^{125}I-labeled Sephadex fraction. The Sephadex fraction was iodinated by the Bolton–Hunter method and subjected to nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension for 2000 V·h on a gradient of pH 3.5–10. The material in the gel was then electrophoresed in the second dimension on a 10% NaDodSO$_4$/polyacrylamide gel. The autoradiogram shows five discrete ^{125}I-labeled spots at 45 kDa (arrowhead). No new spots were seen at 45 kDa when electrophoresis was carried out for 500 or 4000 V·hr. On the right is a one-dimensional gel autoradiogram.

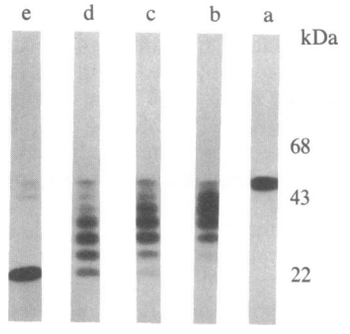

FIG. 5. Endoglycosidase F cleavage of the 45-kDa protein. ^{125}I-labeled 45-kDa protein, purified from a two-dimensional gel (the five 45-kDa spots combined) was treated for 16 hr at 37°C with 0 unit (lane a), 0.025 unit (lane b), 0.25 unit (lane c), 1.0 unit (lane d), and 10.0 units (lane e) of endoglycosidase F. The protein was analyzed by NaDodSO$_4$/10–15% PAGE, and autoradiogram of the dried gel is shown. The exposure time was adjusted in order to visualize the faint bands. Six discrete stages of cleavage are seen. When the protein is cleaved exhaustively, virtually all of the label migrates at 22 kDa (lane e).

The cause of the charge microheterogeneity seen in two-dimensional gel electrophoresis is unknown. It is unlikely that carbohydrate contributes significantly to the generation of five spots because preliminary results with neuraminidase- and endoglycosidase F-treated samples also yield five ^{125}I-labeled spots. Iodination is also unlikely to be the major cause because unlabeled material gives multiple spots as well. Thus, either a different secondary modification, such as phosphorylation or sulfation, or an artifactual modification generated during the purification or analysis may be responsible for the charge heterogeneity (60, 61).

The finding that full biological activity is retained after exhaustive treatment with endoglycosidase F suggests that the carbohydrate residues are not essential for the cholinergic inducing activity of this protein. This contrasts with the result of Weber (40), who found that the activity was destroyed by mild periodate treatment. It is possible that periodate treatment affected the protein core itself, or that the endoglycosidase F incubations did not completely remove the carbohydrate.

The loss of an apparent 23 kDa after deglycosylation suggests the presence of considerable carbohydrate. However, the molecular weight of 45 kDa obtained for both the denatured form (by $NaDodSO_4$/PAGE) and the native form (by Sephadex chromatography) could be an overestimate due to a bulky hydrated structure caused by the presence of at least six carbohydrate chains (62). It is of interest in this regard that Weber has recently obtained an independent estimate of 21 kDa for this or a very similar cholinergic factor based on the sedimentation behavior of the activity in sucrose gradients under native conditions (63).

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